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Dear Editor,

I am pleased to send you a revised version of the manuscript "Chronic treatment with the opioid antagonist naltrexone favours the coupling of spinal cord  $\mu$ -opioid receptors to  $G\alpha_z$  protein subunits" in which the new questions raised by the reviewer 1 have been incorporated, as outlined below in detail. We hope that you will find the revised version suitable for publication in Neuropharmacology.

Thank you for your consideration.

Best regards,

María A Hurlé

Reviewer 1

-Since the authors performed an important positive control experiment (antagonism of delta opioid receptor agonist-mediated activation of G proteins by naltrindole) this figure should be accessible to the readers, maybe as a supplementary figure, rather than indicating "data not shown", p.16 line 8.

The figure was included in the manuscript as supplementary figure S4

-Evidence of downregulation of  $G\alpha_{\text{z}}$  following intracerebroventricular administration of ODN should be shown by western-blotting on spinal cord extracts (to support the behavioral result shown in the new figure 4).

We performed western blotting experiments that shown a down-regulation of  $G\alpha_{\text{z}}$  protein content in the spinal cord from animals treated with  $G\alpha_{\text{z}}$ -ONA (p15, lines 8-10). A new figure (Fig 5) showing these results was included.

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Chronic treatment with the opioid antagonist naltrexone favours the coupling of spinal cord  $\mu$ -opioid receptors to  $G\alpha_z$  protein subunits

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## Abstract

Sustained administration of opioid antagonists to rodents results in an enhanced antinociceptive response to agonists. We investigated the changes in spinal  $\mu$ -opioid receptor signalling underlying this phenomenon. Rats received naltrexone (120  $\mu$ g/h; 7 days) via osmotic minipumps. The antinociceptive response to the  $\mu$ -agonist sufentanil was tested 24 h after naltrexone withdrawal. In spinal cord samples, we determined the interaction of  $\mu$ -receptors with  $G\alpha$  proteins (agonist-stimulated [ $^{35}$ S]GTP $\gamma$ S binding and immunoprecipitation of [ $^{35}$ S]GTP $\gamma$ S-labelled  $G\alpha$  subunits) as well as  $\mu$ -opioid receptor-dependent inhibition of the adenylyl cyclase (AC) activity. Chronic naltrexone treatment augmented DAMGO-stimulated [ $^{35}$ S]GTP $\gamma$ S binding, potentiated the inhibitory effect of DAMGO on the AC/cAMP pathway, and increased the inverse agonist effect of naltrexone on cAMP accumulation. In control rats, the inhibitory effect of DAMGO on cAMP production was antagonized by pertussis toxin (PTX) whereas, after chronic naltrexone, the effect became resistant to the toxin, suggesting a coupling of  $\mu$ -receptors to PTX-insensitive  $G\alpha_z$  subunits. Immunoprecipitation assays confirmed the transduction switch from  $G\alpha_{i/o}$  to  $G\alpha_z$  proteins. The consequence was an enhancement of the antinociceptive response to sufentanil that, in consonance with the neurochemical data, was prevented by  $G\alpha_z$  antisense oligodeoxyribonucleotides but not by PTX. Such changes in opioid receptor signalling can be a double-edged sword. On the one hand, they may have potential applicability to the optimisation of the analgesic effects of opioid drugs for the control of pain. On the other hand, they represent an important

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1 Chronic treatment with the opioid antagonist naltrexone favours the coupling of spinal  
2 cord  $\mu$ -opioid receptors to  $G\alpha_z$  protein subunits

3

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4 receptor signalling underlying this phenomenon. Rats received naltrexone (120  $\mu$ g/h;  
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24 effects in patients chronically treated with opioid antagonists.



1 **Keywords:** Opioid antagonist, Opioid receptors, G-protein, adenylyl cyclase,  
2 constitutive activity, inverse agonism

3

4

5 **Abbreviations:**

6

7 AC: Adenylyl cyclase

8 DAMGO: Tyr-D-Ala-Gly-Me-Phe-Gly-ol-enkephalin

9 FK: Forskolin

10 ODN: oligodeoxyribonucleotides

11 PTX: Pertussis toxin

12

13

## 1 **1. Introduction**

2 Opioid drugs produce their pharmacological effects by interacting with specific G-  
3 protein-coupled receptors (namely  $\mu$ -,  $\delta$ - and  $\kappa$ -opioid receptors) (Snyder and  
4 Pasternak, 2003). The functional interaction of opioid receptors with the pertussis  
5 toxin (PTX)-sensitive  $G\alpha_{i1,2,3}$  and  $G\alpha_o$  transducers as well as PTX-resistant  $G\alpha_z$  has  
6 been clearly demonstrated in heterologous expression systems, neural cell lines and  
7 in the CNS (Chan et al., 1995; Chalecka-Franaszek et al., 2000; Tso and Wong,  
8 2000). In animal models, evidence that  $G\alpha_{i1,2,3}$ ,  $G\alpha_o$ ,  $G\alpha_z$  and  $G\alpha_q$  can contribute to  $\mu$ -  
9 opioid signalling and antinociception was provided by studies using PTX, specific  
10 antisera or antisense oligodeoxyribonucleotides against specific G-protein subunits  
11 and null transgenic mouse strains (Sánchez-Blázquez et al., 1995; Standifer et al.,  
12 1996; Garzón et al., 1998; Sánchez-Blázquez et al., 2001; Hendry et al., 2000; Yang  
13 et al., 2000; Yoburn et al., 2003; Mostany et al., 2008; Lamberts et al., 2011). In  
14 addition to the specificity of receptor-G-protein coupling, different selective agonists  
15 for a specific-receptor can induce different modes of ligand-receptor interaction, and  
16 the particular activation pattern of G-protein subtypes determines the intrinsic activity  
17 of the agonist for the elicited biological response (Garzón et al., 1998; Sánchez-  
18 Blázquez et al., 2001; Valdizán et al., 2010).

19 One of the best characterised effector systems linked to the opioid receptor signalling  
20 cascade is the adenylyl cyclase (AC)/cAMP pathway (Law et al., 2000). Following  
21 receptor activation, opioid drugs exert an inhibitory effect on AC activity through  $G\alpha_i$   
22 subunits, resulting in reduced cAMP production (George et al., 2000; Laugwitz et al.  
23 1993; Mostany et al., 2008). The AC/cAMP pathway has long been known to play a  
24 crucial role in the processing of painful stimuli, and studies have demonstrated an

1 important role of several AC isoforms in inflammatory and neuropathic pain models,  
2 as well as in opioid-induced analgesia (see Pierre et al., 2009).

3 Chronic treatment with opioid ligands (agonists and antagonists) as well as other  
4 non-opioid drugs (for example, calcium channel blockers) critically modifies,  
5 quantitatively and qualitatively, opioid receptor signalling. This modification results in  
6 important changes in the pharmacological potency, efficacy and intrinsic activity of  
7 opioid drugs as well as in the quality of the elicited response (Bannister and  
8 Dickenson, 2010; Chang et al., 2007; Dierssen et al., 1990; Gullapalli and Ramarao,  
9 2002; Hurlé et al., 2000; Mostany et al., 2008; Santillán et al., 1998; Vanderah et al.,  
10 2001). In this context, the development of functional super-sensitivity to opioid  
11 agonists after long-term exposure to opioid receptor antagonists, such as naloxone,  
12 naltrexone and 6 $\beta$ -naltrexol, is a well-known phenomenon in rodents (Sirohi et al.,  
13 2007). For example, we previously reported that the antinociceptive and respiratory  
14 depressant potencies of  $\mu$ -agonists are enhanced following interruption of long-term  
15 treatment with naltrexone in rats (Díaz et al., 2002). In most of the studies in the  
16 literature, this increased responsiveness to agonists has been correlated with opioid  
17 receptor up-regulation (Díaz et al., 2002; Lesscher et al., 2003; Patel et al., 2003;  
18 Sirohi et al., 2007; Unterwald et al., 1995; Yoburn et al., 1986).

19 Sustained opioid receptor blockade by naltrexone is among the currently available  
20 treatments for substance abuse and dependence disorders, and the recently  
21 introduced long-acting, sustained-release formulations of naltrexone are considered  
22 to be promising strategies for the treatment of heroin (Krupitsky and Blokhina, 2010),  
23 alcohol (Anton, 2008; Ray et al., 2010) and nicotine (David et al., 2006) dependence.  
24 However, the advantages and disadvantages of these new therapies have not been  
25 systematically analysed.

1 The neurochemical adaptations produced by continued opioid antagonist treatment  
2 have scarcely been studied. Here, we further analyse the molecular mechanisms  
3 underlying the increased functional responsiveness to opioid agonists produced by  
4 sustained administration of antagonists in rats. We demonstrate that following long-  
5 term treatment with naltrexone, spinal  $\mu$ -opioid receptors undergo a transductional  
6 shift from PTX-sensitive  $G\alpha_{i/o}$  to PTX-resistant  $G\alpha_z$  transducer proteins.  
7 Consequently, the inhibitory effect of agonists on the AC/cAMP effector pathway is  
8 enhanced. In addition, the population of constitutively active  $\mu$ -receptors in the spinal  
9 cord appears to be increased. These neurochemical changes correlate with the  
10 pharmacological super-sensitivity to the antinociceptive effect of the  $\mu$ -opioid agonist  
11 sufentanil.

12

## 13 **2. Material and Methods**

### 14 **2.1. Subjects**

15 The experiments were carried out using male Sprague Dawley rats weighing 250–  
16 300 g (Charles River, Harlan, Barcelona, Spain). The animals were housed in  
17 sawdust-lined cages in an environmentally controlled animal facility at 22°C with a  
18 12:12 h light-dark cycle and food/water provided *ad libitum*. This study was approved  
19 by the Cantabria University Institutional Laboratory Animal Care and Use Committee  
20 and performed in strict accordance with the “European Directive for the Protection of  
21 Vertebrate Animals Used for Experimental and Other Scientific Purposes” (European  
22 Union Directive #86/606/EEC).

23

### 24 **2.2. Pharmacological treatments**

1 Diagrams showing the pharmacological treatment schedules are depicted in  
2 supplementary figure S1. Chronic saline (1  $\mu$ l/h) or naltrexone (120  $\mu$ g/h) infusion  
3 was administered using Alzet 2001 osmotic minipumps (Alza Corporation, Palo Alto,  
4 CA, USA) that were implanted subcutaneously under light ether-induced anaesthesia  
5 (figure S1A). These pumps delivered the solutions at a constant rate of 1  $\mu$ l/h for 7  
6 days. On day 7, the minipumps were removed, and the *in vivo* (antinociceptive  
7 response to the  $\mu$ -opioid receptor agonist sufentanil) or *in vitro* assays  
8 (autoradiographic, [<sup>35</sup>S]GTP $\gamma$ S binding and adenylyl cyclase studies) were carried out  
9 24 h after withdrawal from a chronic saline or naltrexone treatment.

10 To interfere with the expression of G $\alpha_z$  proteins, we used a synthetic antisense  
11 oligodeoxynucleotide (ODN) that has previously been characterised (Sanchez-  
12 Blazquez et al., 1995; Serres et al., 2000). The sequence was 5'-  
13 CGTGATCTCACCCCTTGCTCTCTGCCGGGCCAGT-3'. The ODN was  
14 phosphorothioate-modified at the two bases on each end. The sequence of the  
15 missense oligodeoxynucleotide was 5'-CCCTTATTTACTTTTCGCC-3', and it was  
16 phosphorothioate-modified at positions 5'-CC and GC-3' (Sánchez-Blázquez et al.,  
17 1995; Serres et al., 2000). ODNs (5  $\mu$ g/10  $\mu$ l) were administered twice  
18 intracerebroventricularly (i.c.v) under light isofluorane-induced anaesthesia with a 24-  
19 h interval between administrations (figure S1 B). The G $\alpha_z$ -antisense ODN injection  
20 was performed on days 5 and 7 for the rats receiving chronic naltrexone treatment  
21 (figure S1 C). The rats were challenged with sufentanil 24 h later to test whether the  
22 antinociceptive response elicited by activation of  $\mu$ -opioid receptors was mediated by  
23 G $\alpha_z$ .

24 To prevent the activation of G $\alpha_{i1,2,3}$ / G $\alpha_o$  proteins, PTX was administered (1  $\mu$ g/10  $\mu$ l,  
25 i.c.v.), and the antinociceptive response of sufentanil was tested 48 h later (figure S1

1 D). PTX was injected on day 6 to the rats receiving chronic naltrexone treatment  
2 (figure S1 E).

### 3 **2.3. Evaluation of nociception**

4 The tail-flick test was used to assess the nociceptive threshold. A tail-flick response  
5 was elicited by applying radiant heat to the surface of the tail. The intensity of the  
6 stimulus was adjusted so that control latency was within 3 to 5 sec. A cut-off time of  
7 10 sec was established to avoid permanent injury. Tail-flick latencies were measured  
8 before the drug injection and 30 min after subcutaneous administration of sufentanil  
9 (0.1 or 1 µg/kg; figure S1). This drug administration schedule was based on dose-  
10 response curves obtained in previous studies (Díaz et al., 2002).

### 11 **2.4. Autoradiography of µ-opioid agonist-stimulated [<sup>35</sup>S]GTPγS binding**

12 [<sup>35</sup>S]GTPγS binding using tissue sections was performed as described previously  
13 (Sim et al., 1996; Mostany et al., 2008). Sections were first preincubated in assay  
14 buffer (50 mM Tris-HCl, 3 mM MgCl<sub>2</sub>, 0.2 mM EGTA and 100 mM NaCl; pH 7.4) for  
15 15 min at 25°C, followed by a second 15-min preincubation in the same assay buffer  
16 containing 2 mM GDP and 10 mU/ml adenosine deaminase. Sections were then  
17 incubated for 2 h at 25°C in assay buffer containing 1 mM DTT and 0.04 nM  
18 [<sup>35</sup>S]GTPγS. Consecutive sections were used to define basal binding (in the absence  
19 of the opioid agonist), stimulated binding (in the presence of agonist) and non-  
20 specific binding (without agonist and in the presence of 10 µM GTPγS) (Díaz et al.,  
21 2002). The µ-opioid selective agonist DAMGO was used at concentrations ranging  
22 from 10<sup>-10</sup> to 10<sup>-4</sup> M. After this incubation, slides were rinsed twice in cold Tris buffer  
23 (50 mM Tris-HCl; pH 7.4) for 15 min, dipped in distilled water and dried under an ice-  
24 cold air stream.

1 Tissue sections incubated with [<sup>35</sup>S]GTP $\gamma$ S were exposed to autoradiographic films  
2 (Kodak-MR films, GE Healthcare, Spain) along with [<sup>14</sup>C]-radioactive microscales  
3 (GE Healthcare, Spain). In order to generate the autoradiograms, films were  
4 developed following a 48-h [<sup>35</sup>S]GTP $\gamma$ S binding period. Autoradiographic  
5 densitometry was performed using Scion Image software (Scion Corporation,  
6 Maryland, USA). Autoradiographic values of net agonist-stimulated [<sup>35</sup>S]GTP $\gamma$ S  
7 binding were calculated by subtracting basal binding from agonist-stimulated binding.

## 8 **2.5. Immunoprecipitation of [<sup>35</sup>S]GTP $\gamma$ S-labelled G $\alpha$ subunits**

9 Spinal cord samples were homogenised [1:30 (w/v)] in ice-cold buffer (50 mM Tris-  
10 HCl, 250 mM sucrose, 3 mM MgCl<sub>2</sub>, 1 mM EGTA, and 1 mM DTT; pH 7.4) using a  
11 motor-driven glass Teflon tissue potter (10 strokes, 1500 rpm). The homogenates  
12 were then centrifuged (1500 x g, 5 min, 4°C), and the resulting supernatants were  
13 centrifuged again (14,000 rpm, 15 min, 4°C). Resuspended pellets (500  $\mu$ g  
14 protein/ml/assay) were incubated with 20 nM [<sup>35</sup>S]GTP $\gamma$ S and 10  $\mu$ M DAMGO in a  
15 final volume of 100  $\mu$ l for 30 min at 30°C. Non-specific binding was determined in the  
16 presence of 10  $\mu$ M of GTP $\gamma$ S. Membrane suspensions were then solubilised on ice  
17 with in a solution containing 1% Igepal, 0.5% sodium deoxycholate, 0.1% SDS, 2.5  
18 mM CHAPS, 0.1 mM phenylmethylsulfonylfluoride, 0.01 M aprotinin, 1  $\mu$ g/ml  
19 leupeptin, 1  $\mu$ g/ml pestatin, 1  $\mu$ l/ml antipain, and 10  $\mu$ g/ml chymostatin for 30 min.  
20 Solubilised membranes were incubated for 3 h at room temperature with 15  $\mu$ l of  
21 specific rabbit anti-G $\alpha_o$ , anti-G $\alpha_{i1-2-3}$ , and anti-G $\alpha_z$  antibodies immobilised to  
22 superparamagnetic Dynabeads® Protein A (overnight, 4°C). After three washes with  
23 1 ml of PBS, the beads were pelleted, and the bound radioactivity was counted in 4  
24 ml of Ecolite scintillation cocktail. Antibody specificity was confirmed in our  
25 experimental conditions by western blot analysis, as previously described (Mato et

1 al., 2009). The amount of coupling of  $\mu$ -opioid receptors to the diverse G protein  
2 subunits induced by DAMGO ( $10^{-4}$  M) was expressed as percentage over the basal  
3 values in the absence of the agonist (100%).

#### 4 **2.6. Cyclic AMP assays**

5 AC assays were performed using spinal cord samples as described previously  
6 (Mostany et al., 2008). Samples were homogenised (1:60 weight/volume dilution)  
7 with a Teflon/glass grinder (10 strokes, 800 r.p.m.) in an ice-cold homogenisation  
8 buffer (20 mM Tris-HCl, 1 mM EGTA, 5 mM EDTA, 5 mM DTT, 25  $\mu$ M leupeptin and  
9 300 mM sucrose; pH 7.4). The homogenates were centrifuged at 1,500 x g (5 min at  
10 4°C), and the resulting supernatants were centrifuged at 13,000 x g (15 min at 4°C).  
11 The pellets were resuspended (120  $\mu$ g protein/ml) in assay buffer (80 mM Tris-HCl,  
12 0.2 mM EGTA, 1 mM EDTA, 2 mM  $MgCl_2$ , 100 mM NaCl, 60 mM sucrose, 1 mM  
13 DTT, 10  $\mu$ M GTP, 0.5 mM IBMX, 5 mM phosphocreatine, 50 U/ml creatine  
14 phosphokinase, and 5 U/ml myokinase; pH 7.4) without (basal AC activity) or with 10  
15  $\mu$ M forskolin (FK) (FK-stimulated cAMP accumulation). Opioid receptor-mediated  
16 inhibition of FK-stimulated cAMP accumulation was determined using different  
17 concentrations of the agonist DAMGO ( $10^{-9}$  to  $10^{-4}$  M). To test the effect of PTX on  
18 DAMGO-induced inhibition of FK-stimulated cAMP accumulation, samples were  
19 preincubated for 30 min with or without PTX (1  $\mu$ g/ml) in buffer (25 mM Tris-HCl  
20 buffer containing 0.05% SDS, 10 mM DTT, 1 mM EDTA, 2.5  $\mu$ M NAD, and 10 mM  
21 thymidine; pH 7.4, 30°C). The inverse agonism of naltrexone ( $10^{-7}$  to  $10^{-3}$  M) was  
22 analysed by measuring cAMP accumulation in the absence of NaCl and FK (Mato et  
23 al., 2002). The effects of selective opioid antagonists ( $\mu$ ,  $\beta$ -funaltrexamine;  $\delta$ ,  
24 naltrindole; and  $\kappa$ , *nor*-binaltorphimine) added to the media at a concentration of  $10^{-4}$   
25 M were evaluated.



1 Membranes under the different experimental protocols were preincubated for 5 min  
2 at 37°C, then ATP was added to a final concentration of 200 μM and the mixture was  
3 incubated for 10 min at 37°C. The reaction was stopped by boiling for 5 min, and the  
4 cAMP concentration was determined in a 50 μl sample of the supernatant using a  
5 commercial kit (Cyclic AMP [<sup>3</sup>H] assay system, Amersham Biosciences, Barcelona,  
6 Spain). Each cAMP assay was performed in triplicate, and the results are expressed  
7 as pmol cAMP/min/mg protein.

## 8 **2.7. Drugs and chemicals**

9 Sufentanil was kindly provided by Janssen Cylag, S.A. (Madrid, Spain). DAMGO,  
10 naltrexone and FK were purchased from Sigma (Madrid, Spain). The selective  
11 antagonists of μ-opioid receptors (β-funaltrexamine), δ-opioid receptors (naltrindole)  
12 and κ-opioid receptors (*nor*-binaltorphimine) were obtained from Tocris Bioscience  
13 (Biogen S.L., Madrid, Spain). [<sup>35</sup>S]GTPγS (1250 Ci/mmol) was purchased from Perkin  
14 Elmer (Madrid, Spain). PTX was purchased from Calbiochem (Roche Diagnostics,  
15 Barcelona, Spain). The ODNs were synthesised by Sigma-Genosys Ltd. (Cambridge,  
16 UK). Selective rabbit polyclonal antibodies against Gα<sub>o</sub>, Gα<sub>i1,2,3</sub>, and Gα<sub>z</sub> subunits  
17 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).  
18 Representative immunoblots showing the specificity of the antibodies are shown in  
19 supplementary figure S2.

## 20 **2.8. Data analysis**

21 Data analysis was performed using the GraphPad Prism statistical software package  
22 (GraphPad Software, Inc., San Diego, CA, USA). Data from the [<sup>35</sup>S]GTPγS binding  
23 and AC assays were fitted to sigmoidal concentration-response curves to determine  
24 potency (EC<sub>50</sub> and IC<sub>50</sub>, respectively) and theoretical maximal effect (E<sub>max</sub> and I<sub>max</sub>,  
25 respectively). The EC<sub>50</sub> and IC<sub>50</sub> values were normalised as -logEC<sub>50</sub> (pEC<sub>50</sub>) and -

1 logIC<sub>50</sub> (pIC<sub>50</sub>) for statistical comparison. Data represent the mean ± standard error of  
2 the mean (S.E.M.). Statistical analysis was performed using Student's *t*-tests and  
3 one-way ANOVA followed by the Newman-Keuls *post-hoc* test when appropriate. A  
4 p<0.05 was considered to be statistically significant.

5

### 6 **3. Results**

#### 7 **3.1. Chronic naltrexone treatment increases DAMGO-induced [<sup>35</sup>S]GTPγS** 8 **binding**

9 Basal and agonist-induced μ-opioid receptor activation of G-proteins was determined  
10 by [<sup>35</sup>S]GTPγS binding using spinal cord sections. The basal level of [<sup>35</sup>S]GTPγS  
11 binding was not different between groups. However, animals chronically treated with  
12 naltrexone exhibited a significant increase in μ-opioid receptor-mediated stimulation  
13 of [<sup>35</sup>S]GTPγS binding. The maximal stimulatory effect induced by the selective μ-  
14 opioid agonist DAMGO on spinal cord [<sup>35</sup>S]GTPγS binding was significantly enhanced  
15 in comparison with saline-treated rats, as indicated by the E<sub>max</sub> values determined  
16 from the concentration-response curves. In contrast, the potency between groups  
17 was not different (table 1; figure 1). These data suggest that the μ-opioid receptor  
18 coupling to G proteins was increased following chronic naltrexone treatment.

19 We also confirmed that chronic naltrexone treatment increased the specific binding of  
20 <sup>3</sup>H-DAMGO to μ-opioid receptors (Díaz et al., 2002;), consistent with the reported up-  
21 regulation of μ-opioid receptors induced by chronic naltrexone treatment (Yoburn et  
22 al., 1986; 1995; Unterwald et al., 1995; Lesscher et al., 2003; Patel et al., 2003;  
23 Sirohi et al., 2007).

1 **3.2. Chronic naltrexone treatment causes potentiation of  $\mu$ -opioid agonist-**  
2 **induced inhibition of cAMP accumulation through a mechanism involving PTX-**  
3 **resistant G proteins**

4 Basal AC activity and the ability of the AC activator FK (10  $\mu$ M) to increase cAMP  
5 levels were not altered following long-term administration of naltrexone in comparison  
6 with saline-treated animals (table 1). In control rats, incubation of the spinal cord  
7 membranes with increasing concentrations ( $10^{-9}$  to  $10^{-4}$  M) of the selective  $\mu$ -opioid  
8 agonist DAMGO produced a concentration-dependent inhibition of FK-stimulated  
9 cAMP accumulation. Following long-term treatment with naltrexone, the maximal  
10 ability of DAMGO to inhibit FK-induced cAMP accumulation was significantly  
11 enhanced with no change in potency (table 1). PTX causes the ADP-ribosylation and  
12 inactivation of  $G_{\alpha_{i/o}}$  proteins, with the exception of  $G_{\alpha_z}$  (Casey et al., 1990). The  
13 presence of PTX in the medium did not modify either basal or FK-stimulated cAMP  
14 accumulation (table 1, figure 2). However, in saline-treated animals, the maximal  
15 inhibitory effect of DAMGO was almost completely prevented by PTX pretreatment,  
16 suggesting the involvement of  $G_{\alpha_{i/o}}$  proteins. On the contrary, in the group of animals  
17 chronically treated with naltrexone, PTX did not antagonise the maximal inhibitory  
18 effect of DAMGO on FK-induced cAMP accumulation, suggesting the involvement of  
19  $G_{\alpha_z}$  proteins (figure 2).

20 **3.3. Chronic naltrexone treatment increases  $\mu$ -opioid receptor coupling to  $G_{\alpha_z}$**   
21 **protein subunits**

22 To further assess the existence of specific changes in the coupling of  $\mu$ -opioid  
23 receptors to the PTX-resistant  $G_{\alpha_z}$  subunits, we performed immunoprecipitation of  
24 DAMGO-activated [ $^{35}$ S]GTP $\gamma$ S-labelled  $G_{\alpha}$  protein subunits.

1 In spinal cord homogenates from animals treated with chronic naltrexone, the  
2 coupling of  $\mu$ -opioid receptors to  $G\alpha$  proteins was significantly increased in  
3 comparison with saline-treated animals ( $183.0 \pm 13.3\%$  vs.  $140.1 \pm 9.0\%$  of basal  
4 binding;  $p < 0.05$ ). Western blot analysis of spinal cord samples revealed no change in  
5 the expression of any  $G\alpha$  subunit after chronic naltrexone treatment (see methods  
6 and figure S3 in the supplementary information). However, as shown in figure 3, the  
7 DAMGO-induced activation of  $G\alpha_z$  subunits was significantly increased in chronic  
8 naltrexone-treated animals in comparison with the saline group ( $301.6 \pm 39.9\%$  vs.  
9  $170.7 \pm 10.2\%$  of basal binding;  $p < 0.05$ ). Significant differences in the coupling with  
10  $G\alpha_o$  ( $194.4 \pm 7.7\%$  vs.  $172.6 \pm 5.8\%$ ) and  $G\alpha_{i1-2-3}$  ( $178.9 \pm 7.3\%$  vs.  $160.6 \pm 9.6\%$ )  
11 were not observed between naltrexone- and saline-treated animals.

#### 12 **3.4. Potentiation of $\mu$ -opioid antinociception following withdrawal from chronic** 13 **naltrexone involves $G\alpha_z$ proteins**

14 The functional relevance of the transduction switch from  $G\alpha_{i/o}$  to  $G\alpha_z$  proteins was  
15 assessed by analysing the consequences of PTX or  $G\alpha_z$ -antisense ODN  
16 pretreatment on the antinociceptive response to sufentanil. Under baseline conditions  
17 (Fig. 4A), sufentanil, acutely administered at the dose of  $1 \mu\text{g}/\text{kg}$  ( $n=10$ ), produced an  
18 antinociceptive response that almost reached the MPE; this effect was prevented by  
19 PTX ( $1 \mu\text{g}/10 \mu\text{l}$ , i.c.v.) administered 48 h beforehand ( $n=5$ ). In contrast, pretreatment  
20 with  $G\alpha_z$ -antisense ODN ( $5 \mu\text{g}/10 \mu\text{l}$ , two i.c.v. injections on alternate days;  $n=5$ ) did  
21 not significantly modify the effect of sufentanil. Neither saline nor missense ODN  
22 administered i.c.v. modified the tail-flick basal response or sufentanil-induced  
23 antinociception. These results indicate that sufentanil-induced antinociception in  
24 naïve animals is dependent on the interaction of  $\mu$ -opioid receptors with PTX-  
25 sensitive  $G\alpha_{i/o}$  transducer proteins rather than PTX-insensitive  $G\alpha_z$  subunits.

1 In the rats that received the chronic naltrexone treatment (figure 4B), the  
2 antinociceptive response of sufentanil (0.1  $\mu\text{g}/\text{kg}$ ;  $n = 5$ ) was significantly potentiated,  
3 confirming “in vivo” the development of functional super-sensitivity to the  
4 antinociceptive effect of sufentanil. PTX injected on the 6<sup>th</sup> day of chronic naltrexone  
5 infusion did not prevent the development of opioid super-sensitivity. On the other  
6 hand,  $\text{G}\alpha_z$ -antisense ODN injected i.c.v. on days 5 and 7 of the chronic naltrexone  
7 infusion completely prevented the development of super-sensitivity to the  
8 antinociceptive response elicited by sufentanil. **The reduction in the expression levels  
9 of  $\text{G}\alpha_z$ -proteins in the dorsal horn of the spinal cord induced by  $\text{G}\alpha_z$ -antisense ODN  
10 treatment was confirmed by western blotting experiments (Figure 5).** Overall, these  
11 results indicate that following withdrawal from chronic naltrexone, the antinociceptive  
12 response mediated by  $\mu$ -opioid receptor activation involved  $\text{G}\alpha_z$ -transducer proteins.

### 13 **3.5. Chronic naltrexone treatment increases the constitutive activity of $\mu$ -opioid** 14 **receptors**

15 Incubation of spinal cord membranes from saline-treated rats with increasing  
16 concentrations of naltrexone induced a concentration-dependent increase in the  
17 levels of cAMP ( $E_{\text{max}} = 29.1 \pm 0.7$  pmol/min/mg;  $\text{pEC}_{50} = 4.2 \pm 0.3$ ). This inverse-  
18 agonist action of naltrexone was potentiated after chronic administration of  
19 naltrexone because the maximal cAMP production appeared significantly enhanced  
20 ( $E_{\text{max}} = 35.3 \pm 0.8$ ,  $p < 0.01$  vs. saline-treated group;  $\text{pEC}_{50} = 4.1 \pm 0.2$ ;  $p = \text{NS}$ ),  
21 indicating the existence of constitutively active opioid receptors that uncovered the  
22 inverse-agonist effect of naltrexone (figure 6A).

23 To determine the subtype of opioid receptor that exhibited constitutive activity, the  
24 effect of naltrexone on cAMP levels was evaluated in the presence of selective  
25 antagonists to  $\mu$ - ( $\beta$ -funaltrexamine),  $\delta$ - (naltrindole) and  $\kappa$ - (*nor*-binaltorphimine)

1 receptors at a concentration of  $10^{-4}$  M. The effect of each antagonist alone was  
2 examined in parallel, and only *nor*-binaltrophimine increased cAMP levels (data not  
3 shown), confirming its reported inverse agonism (Wang et al., 2007). As shown in  
4 figure 6B,  $\beta$ -funaltrexamine antagonised the naltrexone-induced cAMP increase both  
5 in the saline-treated group ( $109.1 \pm 2.9\%$  vs.  $121.1 \pm 5.4\%$  in the absence of  $\beta$ -  
6 funaltrexamine;  $p < 0.05$ ) and in the chronic naltrexone-treated group ( $114.4 \pm 0.4\%$   
7 vs.  $147.2 \pm 2.2\%$  in the absence of  $\beta$ -funaltrexamine;  $p < 0.01$ ). Naltrindole ( $10^{-4}$ M) did  
8 not modify the naltrexone-induced cAMP increase in any group. The same  
9 concentration of naltrindole antagonised the binding of [ $^{35}$ S]GTP $\gamma$ S induced by the  $\delta$ -  
10 specific agonist DSLET (Figure S4). *Nor*-binaltrophimine not only was unable to  
11 antagonise but also increased naltrexone-induced cAMP accumulation in both the  
12 saline group ( $142.2 \pm 2.1\%$ ;  $p < 0.01$  vs. the effect of naltrexone alone) and the chronic  
13 naltrexone group ( $165.3 \pm 10.1\%$ ;  $p < 0.05$  vs. the effect of naltrexone alone).  
14 Furthermore, immunoprecipitation assays that were carried out using spinal cord  
15 samples from saline and chronic naltrexone-treated animals demonstrated the  
16 absence of naltrexone-induced coupling with  $G\alpha_s$  ( $104 \pm 8.1\%$ ). Overall, our data  
17 support the interaction of naltrexone with constitutively active  $\mu$ -opioid receptors.

18

#### 19 **4. Discussion**

20 Functional super-sensitivity to opioid agonists induced by sustained exposure to  
21 antagonists is a well-known phenomenon in rodents. Most studies addressing the  
22 underlying mechanisms have focused on the up-regulation of opioid receptors in the  
23 CNS subsequent to blockade (Díaz et al., 2002; Lesscher et al., 2003; Patel et al.,  
24 2003; Sirohi et al., 2007; Unterwald et al., 1995; Yoburn et al., 1986; 1995).

25 Treatment with naltrexone clearly induces the up-regulation of  $\mu$ - and, to a lesser

1 extent,  $\delta$ - and  $\kappa$ -opioid receptors throughout the brain, with differences in the per cent  
2 change across various brain regions (Lesscher et al., 2003; Yoburn et al., 1995).  
3 Furthermore, the increase in the maximal stimulatory effect of DAMGO on the spinal  
4 cord binding of [<sup>35</sup>S]GTP $\gamma$ S indicates the existence of enhanced coupling between  $\mu$ -  
5 opioid receptors and their cognate G-proteins.  
6 Regarding intracellular effectors, one of the best-characterised signalling cascades  
7 linked to opioid receptor activation is the AC/cAMP pathway (Law et al., 2000). This  
8 pathway has long been known to play a crucial role in processing nociception. In  
9 addition to opioids, other pharmacological agents with analgesic properties exert an  
10 inhibitory influence on this pathway (Pierre et al., 2009). In agreement with previous  
11 reports (George et al., 2000; Laugwitz et al. 1993; Mostany et al. 2008), we observed  
12 that PTX-sensitive G $\alpha_i$  subunits were the preferential transducers linking  $\mu$ -opioid  
13 receptor activation to the AC/cAMP pathway in naive animals. Following chronic  
14 naltrexone treatment, the inhibitory effect of DAMGO on the AC/cAMP pathway was  
15 significantly potentiated. However, under these experimental conditions, the effect  
16 was not prevented by PTX, in contrast to the saline-treated group. Thus, our data  
17 indicate that following chronic treatment with antagonists,  $\mu$ -opioid receptors  
18 underwent a shift in the transduction of their signal, showing a higher efficiency of  
19 interaction with PTX-resistant over PTX-sensitive G $\alpha$  proteins. A likely transducer  
20 candidate is G $\alpha_z$ , which is the only G $\alpha$  subunit resistant to PTX (Casey et al., 1990)  
21 that inhibits AC (Kozasa and Gilman, 1995; Mostany et al., 2008). Consistent with  
22 this assumption, the immunoprecipitation data indicated that the coupling of  $\mu$ -opioid  
23 receptors to G $\alpha_z$  subunits was augmented following withdrawal from chronic  
24 naltrexone, whereas the coupling to G $\alpha_o$  and G $\alpha_{i1-2-3}$ , subunits remained similar to  
25 that observed in saline-treated rats.

1 Although the present study provides no information about the mechanisms that could  
2 explain why the switch from  $G\alpha_i$  to  $G\alpha_z$  transducer proteins resulted in an  
3 enhancement of the opioid inhibitory effect on the AC/cAMP effector pathway,  
4 several observations led us to propose some putative mechanisms for such a  
5 phenomenon. First, the rate of  $G\alpha_z$ GTP hydrolysis is as much as 200-fold slower  
6 than that determined for other G protein  $\alpha$  subunits. This extremely slow rate of GTP  
7 hydrolysis would then result in a long-lasting signal (Casey et al., 1990; Jeong and  
8 Ikeda 1998). Second, the inhibitory  $G\alpha$  subunits differ in their specificity for individual  
9 AC isoforms. For example, it has been suggested that the relatively high affinity of  
10  $G\alpha_z$  for AC type V, together with its slow GTPase activity, might account for its  
11 capacity to induce strong AC inhibition in cultured cells (Ammer and Christ, 2002).  
12 Finally,  $G\alpha_z$  may be difficult to switch off after receptor activation unless external  
13 factors, such as RGS-Rz proteins, accelerate the rate of  $G\alpha_z$ GTP hydrolysis. In  
14 particular, RGSZ2 plays an important role in controlling  $\mu$ -opioid signalling induced by  
15  $G\alpha_z$  transducer proteins (Garzón et al., 2005). Thus, it may be feasible that, following  
16 chronic naltrexone treatment, an inadequate control of  $G\alpha_z$  activity may lead to  
17 strong inhibition of the AC/cAMP pathway.

18 The functional relevance of the particular transducer protein linking  $\mu$ -opioid receptor  
19 activation to the AC/cAMP signalling pathway is strengthened by our data that  
20 demonstrate the close relationship between agonist-activated signalling “in vitro” and  
21 agonist-induced pharmacological effects “in vivo”. Thus, as observed in the AC  
22 assay, sufentanil-induced antinociception in naive rats was prevented by PTX but not  
23 by  $G\alpha_z$  antisense ODN, indicating the involvement of  $G\alpha_{i/o}$  transducer proteins. In  
24 contrast, following withdrawal from chronic naltrexone treatment, the switch from  
25  $G\alpha_{i/o}$  to  $G\alpha_z$  proteins appeared to be responsible for the enhanced antinociceptive



1 response to  $\mu$ -opioid agonists because sufentanil-induced antinociception was  
2 prevented by  $G\alpha_z$  antisense ODN but not by PTX pretreatment.

3 Aside from  $G\alpha$  transducer proteins and the AC/cAMP pathway, chronic treatment  
4 with naltrexone could have additional consequences on other elements linked to  $\mu$ -  
5 opioid receptor signalling that were not analysed in this study. In this regard, voltage-  
6 gated  $Ca^{2+}$  channels and G protein-coupled inwardly rectifying  $K^+$  channels are  
7 fundamental determinants of opioid-induced antinociception (Law et al., 2000; Heinke  
8 et al., 2011), whose modulation by  $G\alpha_z$  has been described in several reports (see  
9 Ho and Wong, 2001). Moreover,  $G_{\beta\gamma}$ -subunits broadly regulate Kir3 channels,  
10 voltage-gated  $Ca^{2+}$  channels, phospholipase  $C\beta$ , and several isoforms of AC, among  
11 other effectors (Dupré et al., 2008).

12 In addition to chronic naltrexone treatment, a number of pharmacological  
13 interventions induce analogous signalling plasticity on  $\mu$ -opioid receptors with similar  
14 functional consequences. In this regard, we previously reported that 7 days of  
15 combined treatment with nimodipine (L-type calcium channel blocker) and sufentanil  
16 prevents the development of tolerance and strongly enhances the antinociception in  
17 rats (Dierssen et al., 1990; Hurlé et al., 2000; Mostany et al., 2008). The underlying  
18 mechanism involved efficient inhibition of cAMP production associated with a change  
19 in  $\mu$ -opioid receptor–preferred G-protein coupling from PTX-sensitive  $G\alpha_i$  to PTX-  
20 resistant  $G\alpha_z$  subunits (Mostany et al., 2008). Changes in sensitivity to agonists have  
21 also been reported to occur upon heterodimerisation of opioid receptors. Studies  
22 using cultured cells provide evidence that  $\delta$ -selective antagonists enhance  $\mu$ -opioid  
23 receptor signalling through a mechanism involving the formation of  $\mu$ – $\delta$  hetero-  
24 oligomeric signalling units and a subsequent switch in opioid-receptor preference for  
25  $G\alpha_z$  over  $G\alpha_i$  subunits, which are preferentially activated by individually expressed  $\mu$ -

1 and  $\delta$ -receptors (Fan et al., 2005; George et al., 2000; Hasbi et al., 2007).  
2 Experiments in vivo demonstrate that this change in opioid-receptor transduction  
3 leads to increased  $\mu$ -receptor binding and signalling activity and to an enhancement  
4 of morphine antinociceptive potency in mice (Abul-Husn et al., 2007; Gomes et al.,  
5 2004). Taken together, these findings suggest that conditions favouring the coupling  
6 of  $\mu$ -opioid receptors to  $G\alpha_z$ -protein subunits would increase agonist-induced  
7 AC/cAMP signalling pathways, leading to an enhancement of the pharmacological  
8 responses.

9 Another relevant adaptive response prompted by sustained opioid receptor blockade  
10 arises from the observation that the inverse agonist effect of naltrexone on the AC  
11 activity was significantly potentiated. Opioid receptors, similar to other G-protein  
12 coupled receptors, may exhibit spontaneous constitutive activity even in the absence  
13 of agonists (Sadée et al., 2005). It has also been reported that antagonists, such as  
14 naloxone and naltrexone, display inverse agonist activity when the population of  
15 constitutively active opioid receptors increases, which is typically more prominent  
16 following chronic treatment with opioid agonists (Liu and Prather, 2001; Wang et al.,  
17 2001; Wang et al., 2007). On the other hand, the *in vitro* inverse agonist activity of  
18 naltrexone and other putative  $\mu$ -inverse agonists has been questioned by Divin et al.  
19 2009. These authors observed that, under chronic treatment and the subsequent  
20 rapid removal of opioid agonist, cells expressing  $\mu$ -opioid receptors exhibit an  
21 enhanced cAMP accumulation not linked to the formation of constitutively active  $\mu$ -  
22 opioid receptors.

23 Our present results demonstrate for the first time in native tissue that the inverse  
24 agonism of naltrexone, reflected by cAMP accumulation, occurs after sustained  
25 treatment with opioid antagonists. Furthermore, our findings support the fact that the

1 stimulatory effect of naltrexone on cAMP accumulation was mediated by  $\mu$ -opioid  
2 receptors. In addition, immunoprecipitation assays indicated the lack of involvement  
3 of  $G\alpha_s$  subunits in this effect, demonstrating that naltrexone could not induce the  
4 coupling of  $\mu$ -opioid receptors to these stimulatory subunits. Considering that  
5 receptor over-expression leads to a proportional increase in the number of  
6 spontaneously active receptors (see Leurs et al., 1998), constitutive signalling may  
7 be enhanced after withdrawal from chronic naltrexone treatment as a consequence  
8 of  $\mu$ -opioid receptor up-regulation. However, sensitisation of the receptor to the  
9 effects of inverse agonists cannot be ruled out (Divin et al., 2008; Liu and Prather,  
10 2001; Wang et al., 2007).

11 Interestingly, we observed a potentiation of naltrexone inverse agonism by the  $\kappa$ -  
12 opioid antagonist *nor*-binaltorphimine. In this regard, Wang et al. (2007)  
13 demonstrated that naltrexone has inverse agonist properties at  $\mu$ - but not at  $\delta$ - and  $\kappa$ -  
14 opioid receptors in cultured cells over-expressing opioid receptors. In this study and  
15 in our study (data not shown), *nor*-binaltorphimine exhibited inverse agonist activity at  
16  $\kappa$ -receptors. Thus, such an increase in cAMP accumulation induced by naltrexone in  
17 the presence of *nor*-binaltorphimine may be explained by the sum of their respective  
18 inverse agonist effects on  $\mu$ - and  $\kappa$ -receptors.

## 19 **5. Conclusions**

20 Following long-term treatment with naltrexone,  $\mu$ -receptors in the spinal cord  
21 experienced a transduction shift from PTX-sensitive  $G\alpha_o$  and  $G\alpha_{i1-2-3}$  proteins to PTX-  
22 resistant  $G\alpha_z$  proteins. As a result, the inhibitory effect of the  $\mu$ -agonist DAMGO on  
23 the AC/cAMP effector pathway was enhanced. In addition, constitutively active  $\mu$ -  
24 opioid receptor expression, and possibly  $\kappa$ -opioid receptor expression, in the spinal  
25 cord appeared to be increased. The functional consequence of these neurochemical

1 changes is the development of pharmacological super-sensitivity to the  
2 antinociceptive effect of  $\mu$ -receptor agonists, such as sufentanil. Such changes in  
3 opioid receptor signalling activity can be a double-edged sword. On the one hand,  
4 they may have potential applicability to the optimisation of the analgesic effects of  
5 opioid drugs for the control of pain. On the other hand, they represent an important  
6 homeostatic dysregulation of the endogenous opioid system that might account for  
7 undesirable paradoxical pharmacological effects in patients chronically treated with  
8 certain opioid antagonists.

9

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16

17 **Conflicts of interest:** None

18

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26

1 **Figure legends**

2

3 **Figure 1.** Effect of chronic naltrexone treatment on DAMGO-induced [<sup>35</sup>S]GTP<sub>γ</sub>S  
4 binding. **Left:** Concentration–response curves of DAMGO-stimulated [<sup>35</sup>S]GTP<sub>γ</sub>S  
5 binding using spinal cord sections from animals chronically treated for 7 days with  
6 saline and naltrexone (120 μg/h). Values (mean ± S.E.M.) represent specific  
7 [<sup>35</sup>S]GTP<sub>γ</sub>S binding in nCi/mg tissue. **Right:** Representative autoradiographic  
8 illustrations showing basal (A and B) and 10 μM DAMGO-stimulated (C and D)  
9 [<sup>35</sup>S]GTP<sub>γ</sub>S binding in spinal cord sections from animals chronically treated for 7 days  
10 with saline (A and C) and naltrexone (B and D). Note the enhanced response to  
11 DAMGO following withdrawal from chronic naltrexone in the outer layers of the dorsal  
12 horn (laminae I and II). Abbreviations: DH, dorsal horn; VH, ventral horn.

13

14 **Figure 2.** Effect of pertussis toxin (PTX) on DAMGO-induced inhibition of FK-  
15 stimulated cAMP accumulation in spinal cord homogenates from rats chronically  
16 treated with saline or naltrexone. Data represent the mean ± S.E.M. PTX prevented  
17 opioid-induced inhibition of FK-stimulated cAMP accumulation in spinal cord  
18 homogenates from saline- but not naltrexone-treated rats (\*\**p* < 0.01 and \*\*\**p* < 0.01  
19 vs. FK).

20

21 **Figure 3.** Selective [<sup>35</sup>S]GTP<sub>γ</sub>S labelling of Gα<sub>o</sub>, Gα<sub>1,2,3</sub> and Gα<sub>z</sub> protein subunits  
22 activated by the μ-opioid agonist DAMGO in the spinal cord homogenates from rats  
23 chronically treated with saline or naltrexone. G protein subunits were isolated using  
24 antibodies against each subtype immobilised to superparamagnetic Dynabeads. Data  
25 represent the mean ± S.E.M. of the per cent bound relative to basal binding (100%) for

1 each specific G-protein subunit ( $*p < 0.05$  vs. saline (Newman-Keuls post-ANOVA)).

2 Note the selective increase of [ $^{35}\text{S}$ ]GTP $\gamma$ S labelling of G $\alpha_z$  following chronic

3 administration of naltrexone.

4

5 **Figure 4.** Antinociceptive effect of sufentanil in the tail-flick test. Naive rats (**A**) and  
6 rats chronically treated with naltrexone (**B**) were challenged with sufentanil after i.c.v.  
7 pretreatment with saline, pertussis toxin (PTX; 1  $\mu\text{g}/10 \mu\text{l}$ ), G $\alpha_z$  antisense  
8 oligodeoxynucleotide (ODN; 2 x 5  $\mu\text{g}/10 \mu\text{l}$ ) or missense ODN (2 x 5  $\mu\text{g}/10 \mu\text{l}$ ). Data  
9 represent the mean  $\pm$  S.E.M. of the per cent antinociception relative to the maximal  
10 possible effect (100%) ( $**p < 0.01$  vs. saline;  $***p < 0.001$  vs. baseline;  $###p < 0.001$   
11 vs. saline (Newman-Keuls post-ANOVA).

12

13 **Figure 5.** Effect of G $\alpha_z$  antisense oligodeoxynucleotide on the levels of G $\alpha_z$  proteins  
14 in the dorsal horn of the spinal cord. Representative immunoblots and integrated  
15 optical density (OD) of the bands show a down-regulation of G $\alpha_z$  proteins following  
16 antisense ODN, both in saline and naltrexone (NTX) treated rats. Data represent the  
17 mean  $\pm$  S.E.M. from four animals per group of the per cent OD relative to missense  
18 ODN. ( $*p < 0.05$  vs missense ODN; Newman-Keuls post-ANOVA).

19

20 **Figure 6.** Naltrexone-induced cAMP accumulation in spinal cord homogenates from  
21 animals chronically treated for 7 days with saline or naltrexone (120  $\mu\text{g}/\text{h}$ ) and  
22 selective opioid antagonists. (**A**): Concentration–response curves of naltrexone-  
23 induced cAMP accumulation (pmol/min/mg protein); (**B**) Antagonism of naltrexone-  
24 induced cAMP accumulation by the selective antagonists of  $\mu$ - ( $\beta$ -funaltrexamine),  $\delta$ -  
25 (naltrindole) and  $\kappa$ - (*nor*-binaltrophimine) receptors (percentage with respect to the

1 basal value, 100%). Data represent the mean  $\pm$  S.E.M. Note the increase in the  
2 naltrexone inverse agonist effect following chronic administration of the opioid  
3 antagonist, which is completely abolished by treatment with the selective  $\mu$ -opioid  
4 antagonist  $\beta$ -funaltrexamine, supporting the interaction of naltrexone with  
5 constitutively active  $\mu$ -opioid receptors ( $*p < 0.05$ ,  $**p < 0.01$  and  $***p < 0.001$  vs.  
6 basal value of cAMP level (Newman-Keuls post-ANOVA);  $+p < 0.05$  and  $++p < 0.01$   
7 vs. naltrexone (Newman-Keuls post-ANOVA).

**Table 1**

Effect of chronic administration of naltrexone (120  $\mu\text{g/hr}$ , 7 days) in  $\mu$ -opioid receptor density and functionality, and on adenylyl cyclase (AC) activity

| Type of assay   | Saline             | Chronic naltrexone  |
|---|--------------------|---------------------|
| <i>Autoradiographic density of [<sup>3</sup>H]-DAMGO binding (fmol/mg tissue)</i> | 42.42 $\pm$ 4.15   | 75.42 $\pm$ 7.52**  |
| <i>[<sup>35</sup>S]GTP<math>\gamma</math>S binding autoradiography</i>            |                    |                     |
| Basal binding (nCi/mg tissue)   | 250.22 $\pm$ 40.12 | 278.23 $\pm$ 24.12  |
| DAMGO-stimulated binding  |                    |                     |
| Emax (nCi/mg tissue)  | 538.20 $\pm$ 48.01 | 736.5 $\pm$ 39.75** |
| pEC <sub>50</sub> (-log EC <sub>50</sub> )  | 5.89 $\pm$ 0.35    | 5.86 $\pm$ 0.14     |
| <i>Adenylyl cyclase (AC) activity</i>   |                    |                     |
| Basal AC activity (pmol/min/mg protein)   | 21.30 $\pm$ 3.90   | 27.50 $\pm$ 3.79    |
| Forskolin-induced cAMP accumulation (pmol/min/mg protein)                         | 599.61 $\pm$ 25.34 | 553.98 $\pm$ 43.79  |
| DAMGO inhibition  | 462,42 $\pm$ 14.28 | 389.44 $\pm$ 29,36* |
| Imax (pmol/min/mg protein)  | 6.48 $\pm$ 0.47    | 6.88 $\pm$ 0.16     |
| pIC <sub>50</sub> (-log IC <sub>50</sub> )  |                    |                     |

Emax represents DAMGO-induced maximal stimulation of [<sup>35</sup>S]GTP $\gamma$ S binding; Imax represents DAMGO-induced maximal inhibition of forskolin-induced cAMP-accumulation. Values are given as means $\pm$ S.E.M. of data from 6-7 animals/group. \*p< 0.05 and \*\*p< 0.01 vs saline-treated group (un-paired *t* test).



Figure 1  
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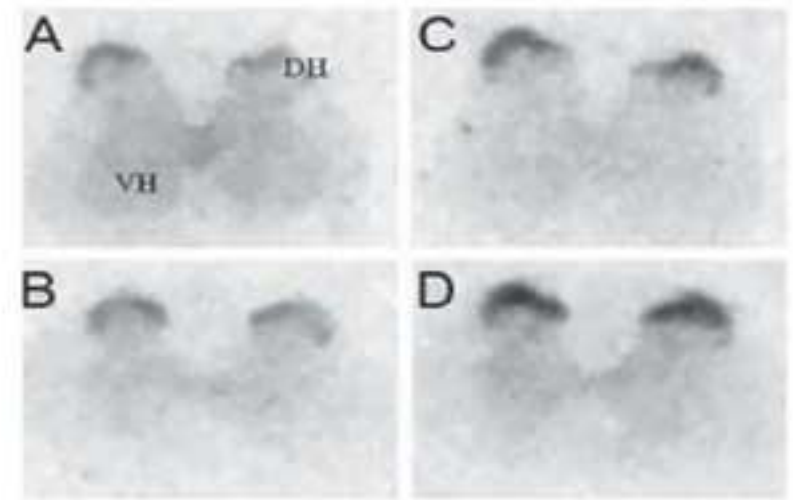
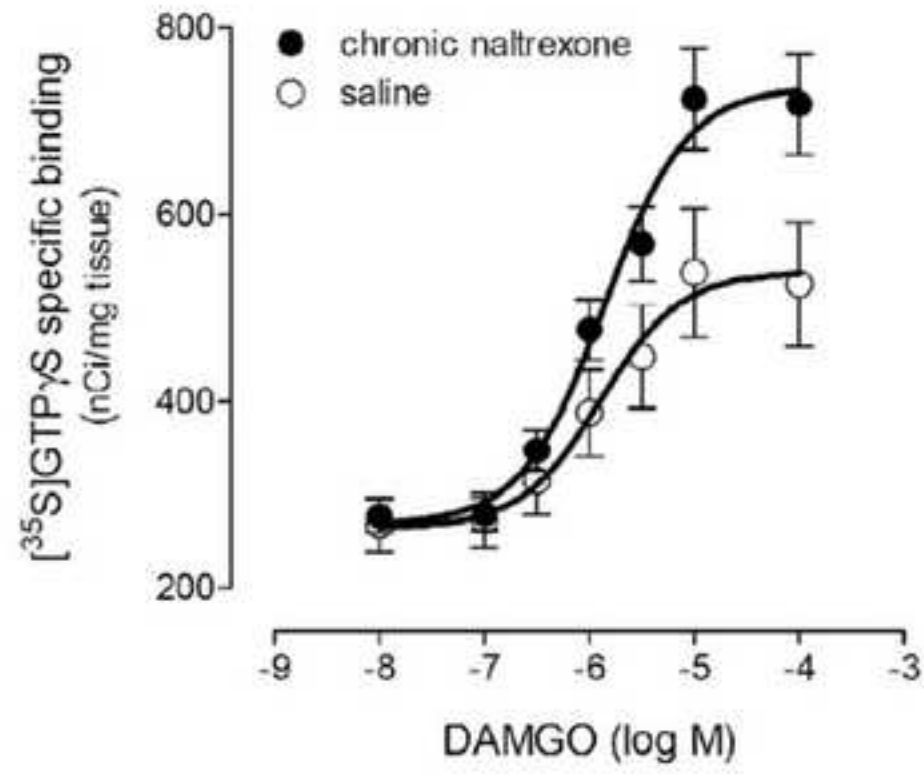


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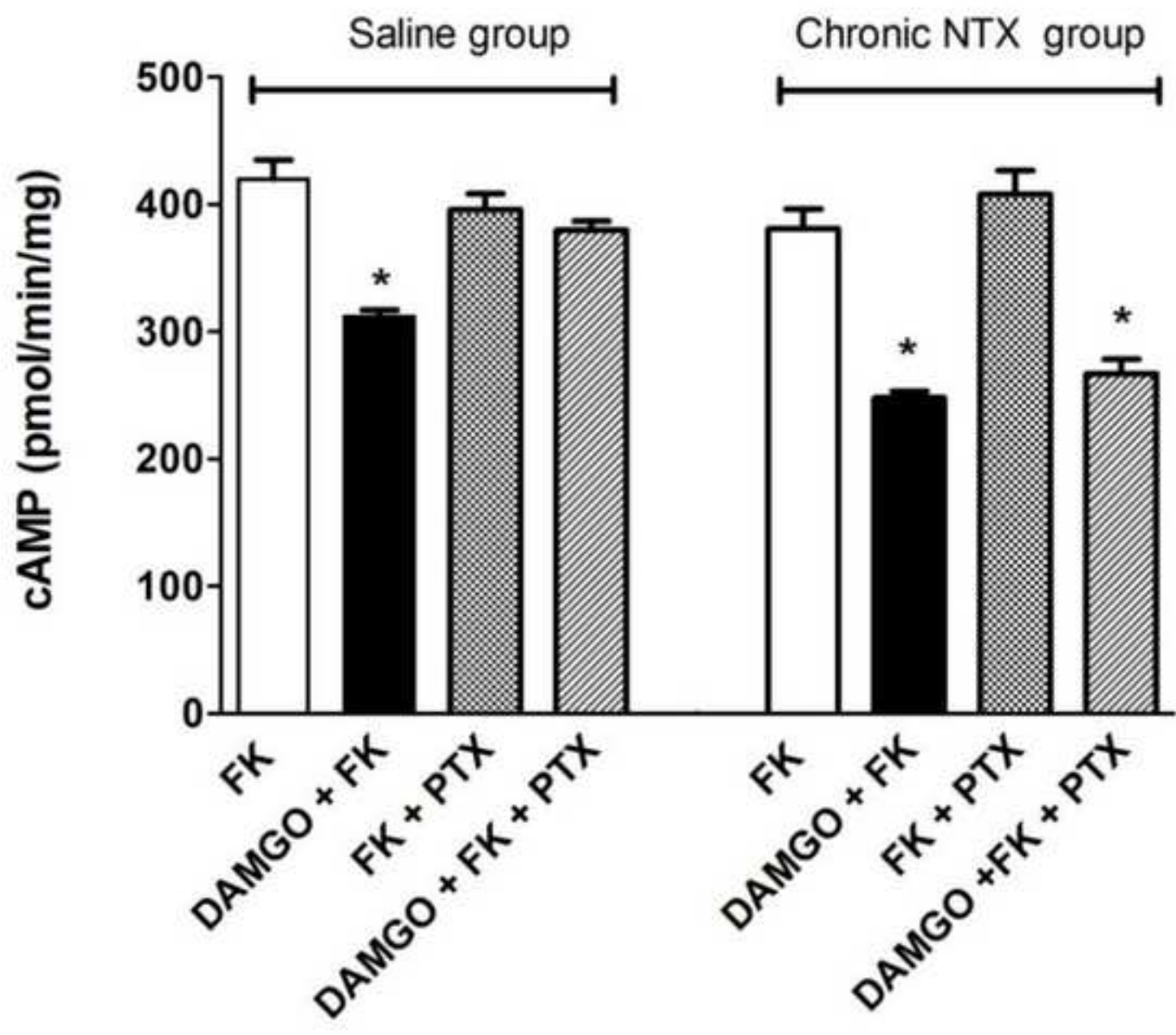


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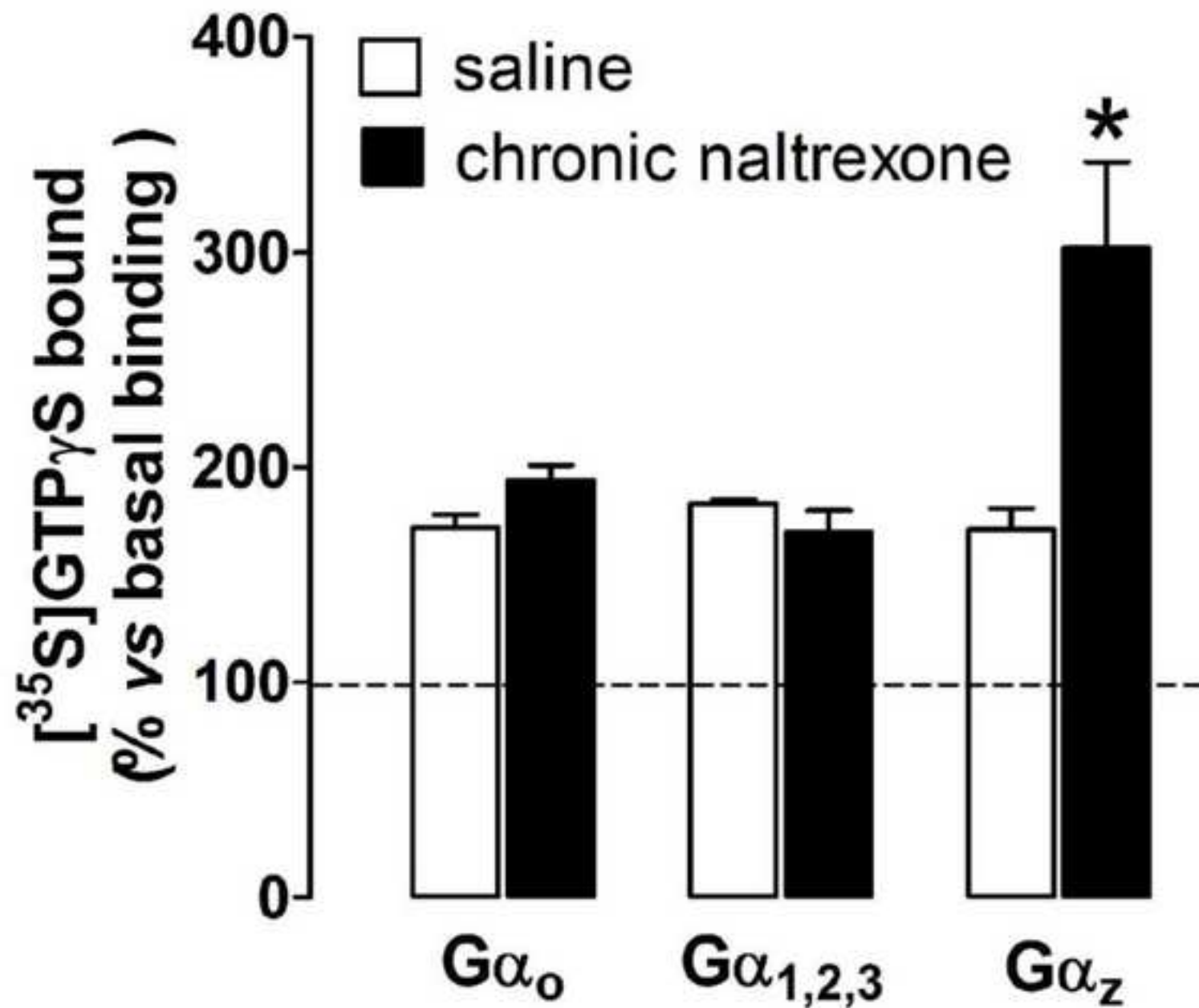


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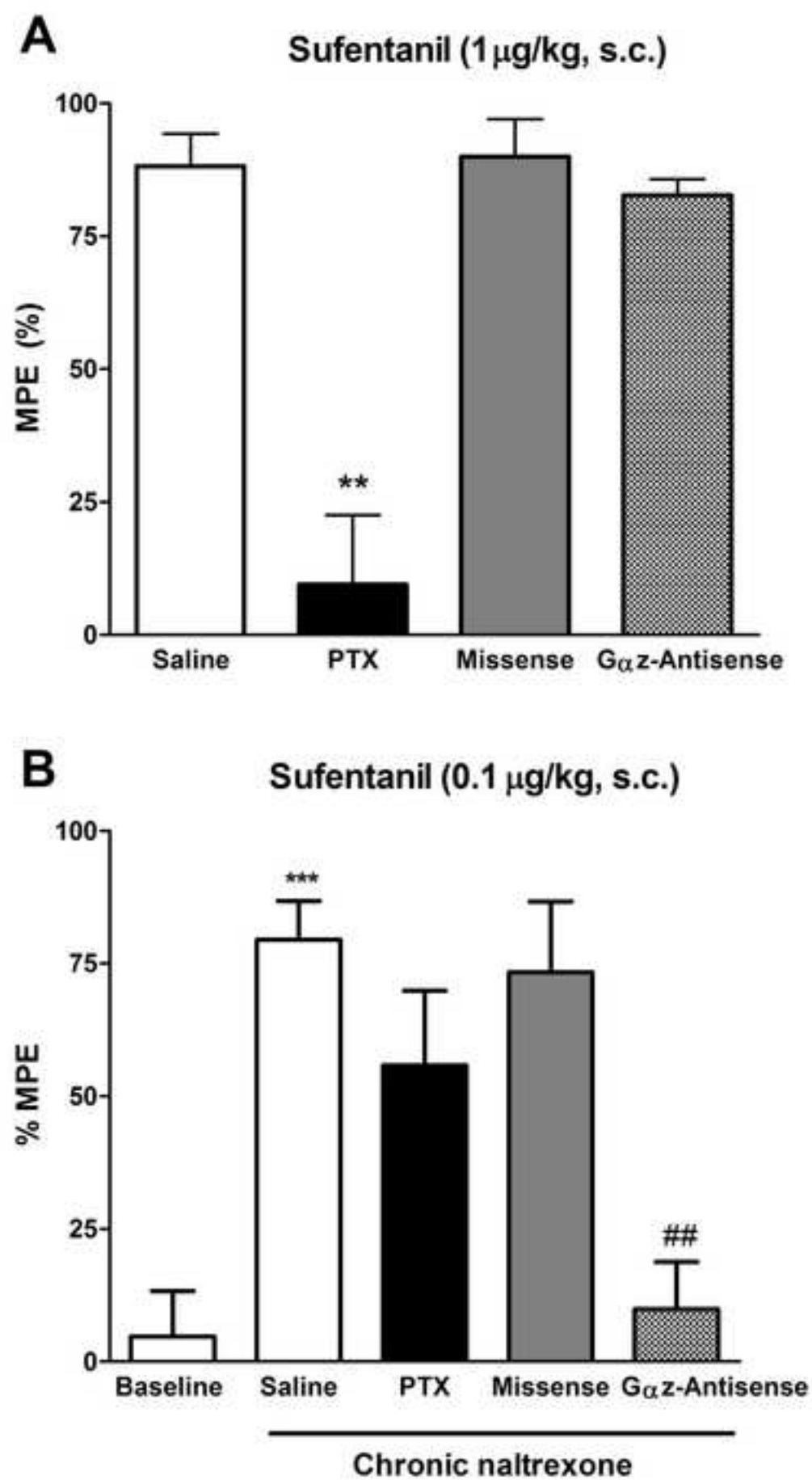


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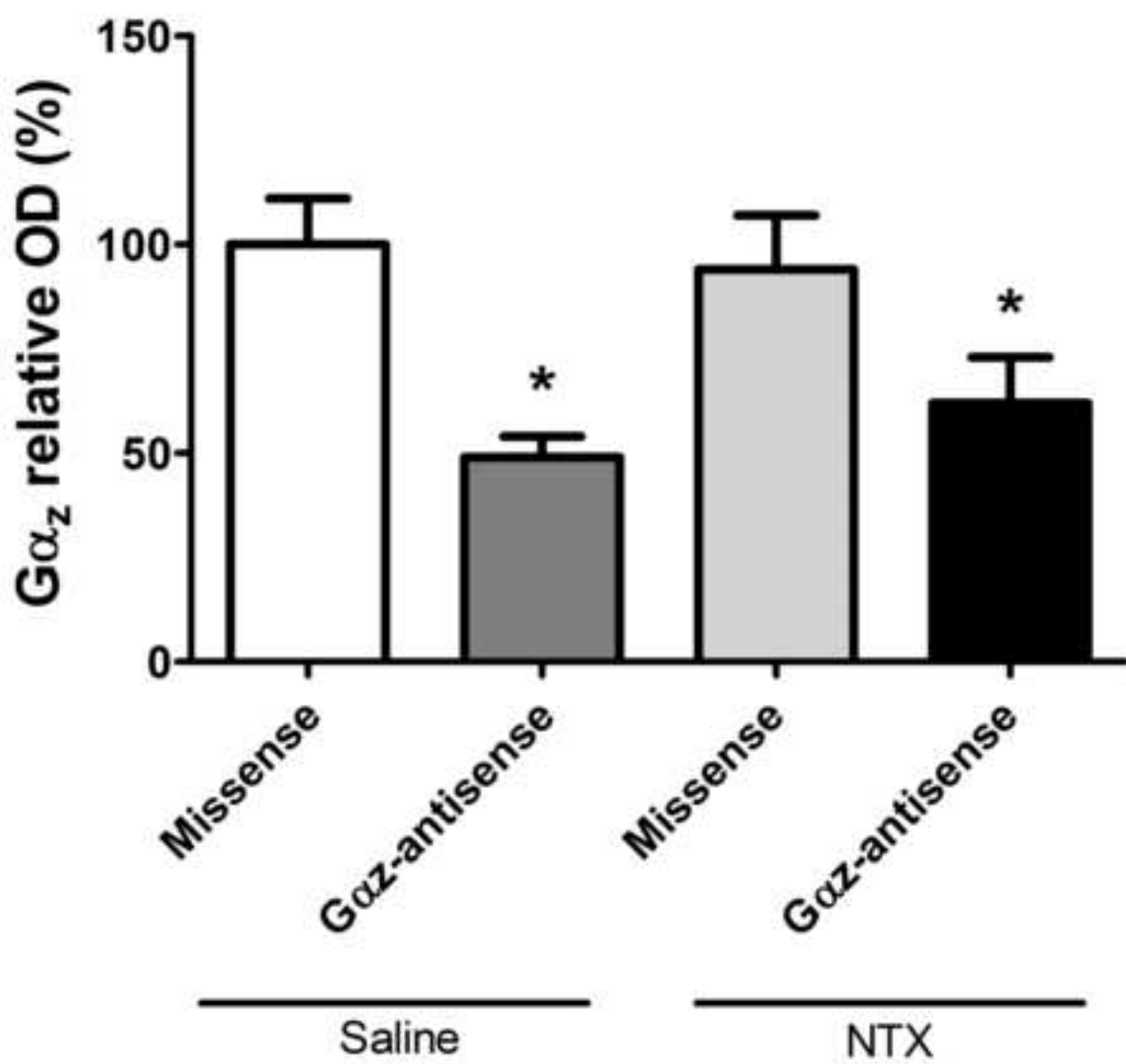
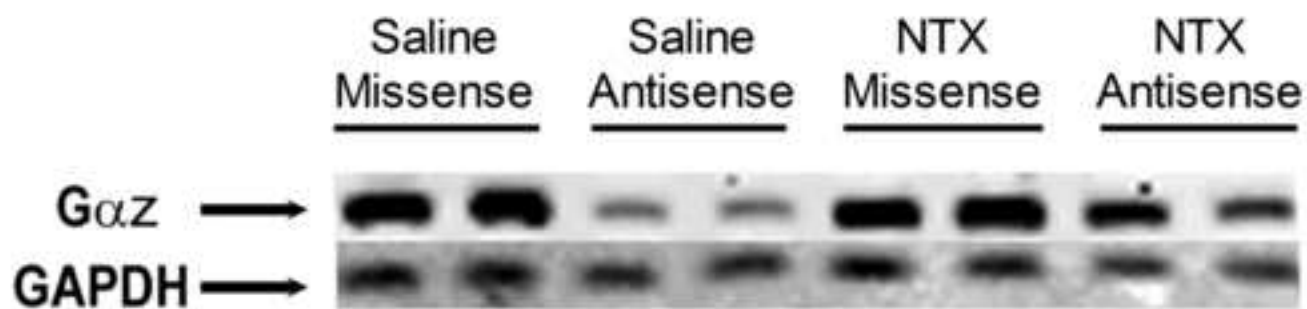
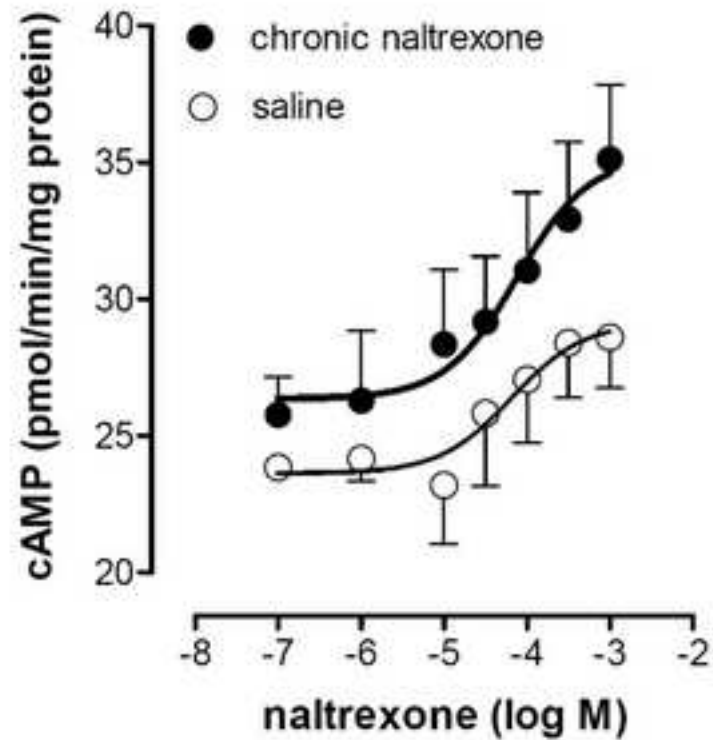


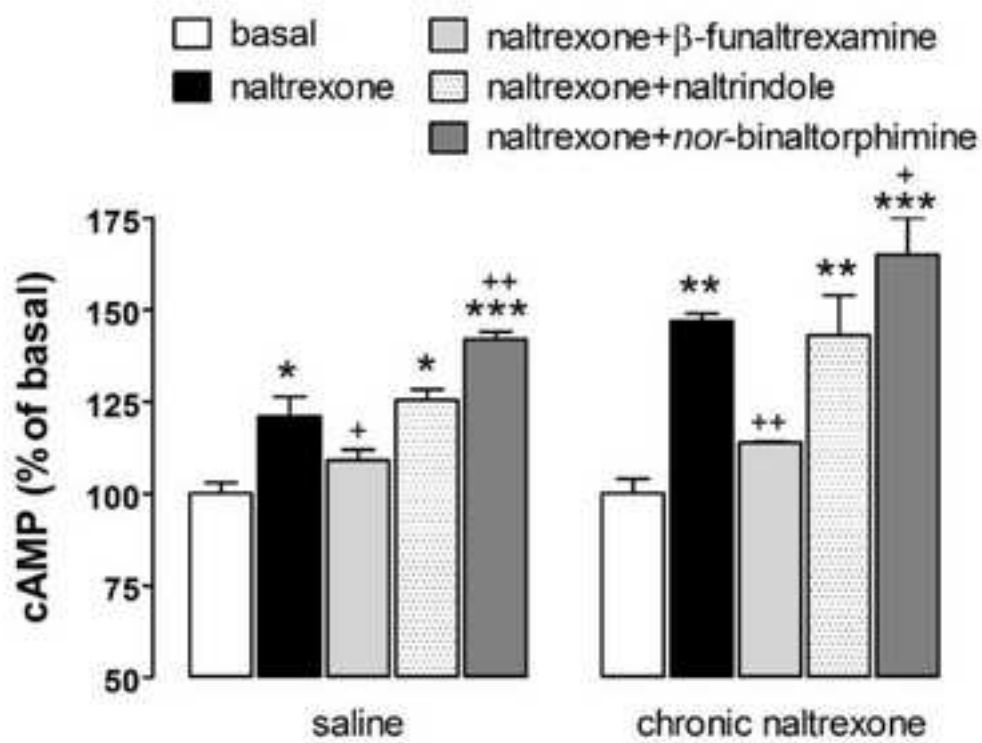
Figure 6

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**A**



**B**



**Supplementary Material**

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## Highlights

1. Chronic treatment with opioid antagonists increases agonist-mediated antinociception
2. We examine spinal cord changes in  $\mu$ -opioid receptor signalling underlying this effect
3. Opioid receptor-coupling to G-proteins and inhibition of cAMP pathway are enhanced
4.  $\mu$ -opioid receptors experience a transduction shift from  $G\alpha_{i/o}$  to  $G\alpha_z$  proteins
5. The inverse agonist effect of naltrexone on cAMP accumulation is enhanced